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=> s "lactic acid bacteria"  
L1 26523 "LACTIC ACID BACTERIA"

=> s "lactic acid bacteria" and enterocyte and "nitric oxide"  
L2 1 "LACTIC ACID BACTERIA" AND ENTEROCYTE AND "NITRIC OXIDE"

TI **Lactic acid bacteria** isolated from dairy products inhibit genotoxic effect of 4-nitroquinoline-1-oxide in SOS-chromotest.

AB Antigenotoxic activity against 4-nitroquinoline-1-oxide (4-NQO) of **lactic acid bacteria** isolated from commercial dairy products was studied using SOS-Chromotest. The supernatants from bacteria-genotoxin co-incubations in general exhibited a strong suppression. . . presented some relevant functional properties, such as tolerance to bile (0.5%) or acid environment (pH 2.0) and adherence to Caco-2 **enterocytes**. Antigenotoxicity was always associated with modification of the 4-NQO absorbance profile.

CT Check Tags: Human; Support, Non-U.S. Gov't  
 4-Nitroquinoline-1-oxide: CH, chemistry  
 \*4-Nitroquinoline-1-oxide: TO, toxicity  
 Bifidobacterium: GE, genetics  
 Bifidobacterium: IP, isolation & purification  
 \*Bifidobacterium: PH, physiology  
 Caco-2 Cells  
 Culture Media  
 \*Dairy Products: MI, . . .

RN 56-57-5 (4-Nitroquinoline-1-oxide)  
 AN 2003072708 MEDLINE  
 DN 22471239 PubMed ID: 12583707

TI **Lactic acid bacteria** isolated from dairy products inhibit genotoxic effect of 4-nitroquinoline-1-oxide in SOS-chromotest.

AU Cenci Giovanni; Rossi Jone; Trotta Francesca; Caldini Giovanna  
 CS Dipartimento di Biologia Cellulare e Molecolare, Universita di Perugia, Italy.. gcenci@unipg.it  
 SO SYSTEMATIC AND APPLIED MICROBIOLOGY, (2002 Dec) 25 (4) 483-90.  
 Journal code: 8306133. ISSN: 0723-2020.  
 CY Germany: Germany, Federal Republic of  
 DT Journal; Article; (JOURNAL ARTICLE)  
 LA English  
 FS Priority Journals  
 EM 200304  
 ED Entered STN: 20030214  
 Last Updated on STN: 20030429  
 Entered Medline: 20030428

L5 ANSWER 2 OF 3 WPIDS COPYRIGHT 2003 THOMSON DERWENT on STN

TI Using **lactic acid bacteria** to regulate intestinal inflammation and immunity, reduces production of **nitric oxide** by activated **enterocytes**.

AB FR 2785809 UPAB: 20000712  
 NOVELTY - Use of a strain (A) of **lactic acid bacteria** (LAB) that can reduce production of **nitric oxide** by **enterocyte** cultures, preactivated by pro-inflammatory cytokines and bacterial lipopolysaccharides (LPS), for preparation of a composition for regulating the inflammatory response of **enterocytes** is new.  
 DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for screening new strains of LAB, having non-specific immunomodulating properties, by testing for inhibition of **nitric oxide** in preactivated **enterocyte** cultures.  
 ACTIVITY - Immunomodulatory; antimicrobial; anti-inflammatory.  
 MECHANISM OF ACTION - (A) inhibits specifically production of **nitric oxide** (which is protective (antimicrobial) for the intestinal mucosa at low levels but at high levels damages epithelial cells and contributes to chronic inflammation) by activated **enterocytes**.  
 USE - (A) are used to regulate the inflammatory response of the intestinal mucosa and non-specific immunity, e.g. for. . . caused by bacterial, fungal or viral infection.

ADVANTAGE - (A) is specific for activated cells, having no effect on nitric oxide production by basal cells.  
Dwg.0/4

TECH. 20000712

TECHNOLOGY FOCUS - BIOLOGY - Preferred Process: The strain is also tested for (i) ability to increase production of nitric oxide by cultures of enterocytes preactivated with pro-inflammatory cytokines only and (ii) optionally also for having no effect on nitric oxide production in non-activated enterocytes. The cells being screened are lactobacilli, lactococci, streptococci and bifidobacteria, most particularly Lactobacillus casei.

TECHNOLOGY FOCUS - PHARMACEUTICALS - Preferred. . .

TT TT: LACTIC ACID BACTERIA REGULATE INTESTINAL INFLAMMATION IMMUNE REDUCE PRODUCE NITRIC OXIDE ACTIVATE.

AN 2000-378538 [33] WPIDS

DNC C2000-114761

TI Using lactic acid bacteria to regulate intestinal inflammation and immunity, reduces production of nitric oxide by activated enterocytes.

DC B04 D13 D16

IN CAYUELA, C; DUGAS, N; POSTAIRE, E

PA (DANO-N) CIE DANONE SA GERVAIS

CYC 91

PI FR 2785809 A1 20000519 (200033)\* 19p

WO 2000028943 A2 20000525 (200033) FR

RW: AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW NL  
OA PT SD SE SL SZ TZ UG ZW

W: AE AL AM AU AZ BA BB BG BR BY CA CN CR CU CZ DM EE GD GE GH GM HR  
HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LV MA MD MG MK MN  
MW MX NO NZ PL RO RU SD SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN  
YU ZA ZW

AU 2000012763 A 20000605 (200042)

EP 1131080 A2 20010912 (200155) FR

R: AL AT BE CH CY DE DK ES FI FR GB GR IE IT LI LT LU LV MC MK NL PT  
RO SE SI

EP 1131080 B1 20030910 (200360) FR

R: AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

DE 69911237 E 20031016 (200376)

ADT FR 2785809 A1 FR 1998-14471 19981118; WO 2000028943 A2 WO 1999-FR2826 19991118; AU 2000012763 A AU 2000-12763 19991118; EP 1131080 A2 EP 1999-956072 19991118; WO 1999-FR2826 19991118; EP 1131080 B1 EP 1999-956072 19991118; WO 1999-FR2826 19991118; DE 69911237 E DE 1999-611237 19991118; EP 1999-956072 19991118; WO 1999-FR2826 19991118

FDT AU 2000012763 A Based on WO 2000028943; EP 1131080 A2 Based on WO 2000028943; EP 1131080 B1 Based on WO 2000028943; DE 69911237 E Based on EP 1131080, Based on WO 2000028943

PRAI FR 1998-14471 19981118

L5 ANSWER 3 OF 3 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN

AB . . . provide excellent therapeutic benefits, The biological activity of probiotic bacteria is due in part to their ability to attach to enterocytes. This inhibits the binding of enteric pathogens by a process of competitive exclusion, Attachment of probiotic bacteria to cell surface receptors of enterocytes also initiates signalling events that result in the synthesis of cytokines, Probiotic bacteria also exert an influence on commensal micro-organisms. . . also alter the ecological balance of enteric commensals. Production of butyric acid by some probiotic bacteria affects the turnover of enterocytes and neutralizes the activity of dietary carcinogens, such as nitrosamines. that are generated by the metabolic activity of commensal bacteria in subjects consuming a high-protein diet. Therefore, inclusion of probiotic. . .

STP KeyWords Plus (R): LACTIC-ACID BACTERIA;

DIETARY MODULATION; TRAVELERS DIARRHEA; YOGURT; MILK; GG; GROWTH;  
PREVENTION; CULTURES; INTOLERANCE

AN 2000:167860 SCISEARCH

GA The Genuine Article (R) Number: 286TE

TI Survival and therapeutic potential of probiotic organisms with reference  
to Lactobacillus acidophilus and Bifidobacterium spp.

AU Kailasapathy K (Reprint); Chin J

CS LOCKED BAG 1, RICHMOND, NSW, AUSTRALIA (Reprint); UNIV WESTERN SYDNEY, CTR  
ADV FOOD RES, HAWKESBURY, NSW, AUSTRALIA; ELIZABETH MACARTHUR AGR INST,  
CAMDEN, NSW, AUSTRALIA

CYA AUSTRALIA

SO IMMUNOLOGY AND CELL BIOLOGY, (FEB 2000) Vol. 78, No. 1, pp. 80-88.

Publisher: BLACKWELL SCIENCE ASIA, 54 UNIVERSITY ST, P O BOX 378, CARLTON  
VICTORIA 3053, AUSTRALIA.

ISSN: 0818-9641.

DT General Review; Journal

FS LIFE

LA English

REC Reference Count: 101

\*ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS\*

=>

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L2: Entry 1 of 24

File: PGPB

Jun 19, 2003

DOCUMENT-IDENTIFIER: US 20030113306 A1

TITLE: Probiotic lactobacillus casei strains

Detail Description Paragraph (95):

[0133] The introduction of probiotic organisms is accomplished by the ingestion of the micro-organism in a suitable carrier. It would be advantageous to provide a medium that would promote the growth of these probiotic strains in the large bowel. The addition of one or more oligosaccharides, polysaccharides, or other prebiotics enhances the growth of lactic acid bacteria in the gastrointestinal tract. Prebiotics refers to any non-viable food component that is specifically fermented in the colon by indigenous bacteria thought to be of positive value, e.g. bifidobacteria, lactobacilli. Types of prebiotics may include those that contain fructose, xylose, soya, galactose, glucose and mannose. The combined administration of a probiotic strain with one or more prebiotic compounds may enhance the growth of the administered probiotic in vivo resulting in a more pronounced health benefit, and is termed synbiotic.

Detail Description Paragraph (121):

[0158] 22. Chauviere, G., M. H. Cocconier, S. Kerneis, J. Fourniat and A. L. Servin. Adherence of human Lactobacillus acidophilus strains LB to human enterocyte-like Caco-2 cells. J Gen. Microbiol. 1992; 138: 1689-1696

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### Abstract

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#### Cellular Microbiology

Volume 5 Issue 4 Page 277 - April 2003

doi:10.1046/j.1462-5822.2003.t01-1-00275.x

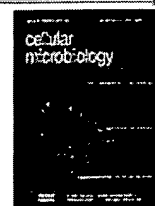
## Lactobacillus rhamnosus GG decreases TNF- $\alpha$ production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism

Jeremy Andrew Peña<sup>1,3</sup> and James Versalovic<sup>1,2,3\*</sup>

### Summary

Animal studies and human clinical trials have shown that *Lactobacillus* can prevent or ameliorate inflammation in chronic colitis. However, molecular mechanisms for this effect have not been clearly elucidated. We hypothesize that lactobacilli are capable of downregulating pro-inflammatory cytokine responses induced by the enteric microbiota. We investigated whether lactobacilli diminish production of tumour necrosis factor alpha (TNF- $\alpha$ ) by the murine macrophage line, RAW 264.7 gamma (NO-), and alter the TNF- $\alpha$ /interleukin-10 (IL-10) balance, *in vitro*. When media conditioned by *Lactobacillus rhamnosus* GG (LGG) are co-incubated with lipopolysaccharide (LPS) or lipoteichoic acid (LTA), TNF- $\alpha$  production is significantly inhibited compared to controls, whereas IL-10 synthesis is unaffected. Interestingly, LGG-conditioned media also decreases TNF- $\alpha$  production of *Helicobacter*-conditioned media-activated peritoneal macrophages. *Lactobacillus* species may be capable of producing soluble molecules that inhibit TNF- $\alpha$  production in activated macrophages. As overproduction of pro-inflammatory cytokines, especially TNF- $\alpha$ , is implicated in pathogenesis of chronic intestinal inflammation, enteric *Lactobacillus*-mediated inhibition of pro-inflammatory cytokine production and alteration of cytokine profiles may highlight an important immunomodulatory role for commensal bacteria in the gastrointestinal tract.

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☐ Jeremy Andrew Peña  
☐ James Versalovic

Received 19 September, 2002; revised 10 January, 2003; accepted 14 January, 2003.

#### Affiliations

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#### Correspondence

\*For correspondence.  
 E-mail: jxversal@texaschildrenshospital.org;  
 Tel. (+1) 832 8242213;  
 Fax (+1) 832 8251032.

**To cite this article** Peña, Jeremy Andrew & Versalovic, James (2003) *Lactobacillus rhamnosus* GG decreases TNF- $\alpha$  production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. *Cellular Microbiology* 5 (4), 277-285. doi: 10.1046/j.1462-5822.2003.t01-1-00275.x

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Terminally differentiated cells comprising the majority of the external surface of the intestinal epithelium (see **INTESTINAL MUCOSA**). Unlike **GOBLET CELLS**, they do not produce or secrete mucins, nor do they secrete cryptdins as do the **PANETH CELLS**.

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**MeSH Tree 1**

- ▶ All MeSH Categories
  - ▶ Anatomy (MeSH Category)
    - ▶ Cells
      - ▶ Epithelial Cells
        - ▶ Ameloblasts
        - ▶ Chief Cells, Gastric
        - ▶ Chromatophores
        - ▶ Dendritic Cells
        - ▶ Enterochromaffin Cells
        - ▶ Enterochromaffin-like Cells
        - ▼ **Enterocytes**
          - ▶ Goblet Cells
          - ▶ Granulosa Cells
          - ▶ Hepatocytes
          - ▶ Keratinocytes
          - ▶ Labyrinth Supporting Cells
          - ▶ Melanocytes
          - ▶ Merkel Cells
          - ▶ Paneth Cells
          - ▶ Parietal Cells, Gastric
          - ▶ Sertoli Cells

---

### MeSH Tree 2

- ▶ All MeSH Categories
  - ▶ Anatomy (MeSH Category)
    - ▶ Digestive System
      - ▶ Gastrointestinal System
        - ▶ Intestines
          - ▶ Intestinal Mucosa
            - ▼ **Enterocytes**
              - ▶ Goblet Cells
              - ▶ Paneth Cells

---

### MeSH Tree 3

- ▶ All MeSH Categories
  - ▶ Anatomy (MeSH Category)
    - ▶ Tissues
      - ▶ Membranes
        - ▶ Mucous Membrane
          - ▶ Intestinal Mucosa
            - ▼ Enterocytes
              - ▶ Goblet Cells
              - ▶ Paneth Cells

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*Am J Physiol Lung Cell Mol Physiol* 268: L501-L508, 1995;  
1040-0605/95 \$5.00

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## ARTICLES

# Pulmonary alveolar epithelial inducible NO synthase gene expression: regulation by inflammatory mediators

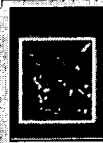
**H. H. Gutierrez, B. R. Pitt, M. Schwarz, S. C. Watkins, C. Lowenstein, I. Caniggia, P. Chumley and B. A. Freeman**

Department of Pediatrics, University of Alabama at Birmingham 35233.

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Nitric oxide (.NO) is a short-lived mediator that can be induced by different cytokines and lipopolysaccharide (LPS) in a variety of cell types and produces many physiological and metabolic changes in target cells. In the current study, we show that a combination of cytokines, LPS, and zymosan-activated serum (ZAS; called for convenience cytomix Z) induces production of high concentrations of the NO oxidation products nitrite (NO<sub>2</sub>-) and nitrate (NO<sub>3</sub>-) by cultured rat fetal lung epithelial type II cells in a time-dependent fashion. Interferon-gamma and tumor necrosis factor-alpha alone did not significantly affect .NO synthesis, whereas ZAS, LPS, and interleukin-1 beta caused only a modest increase in formation of .NO oxidation products. Production of NO<sub>2</sub>- and NO<sub>3</sub>- was inhibited by NG-monomethyl-L-arginine and cycloheximide. After exposure of these cells to a combination of the above cytokines, Escherichia coli LPS, and ZAS (cytomix Z), enhanced inducible nitric oxide synthase (iNOS) expression was indicated by an elevation in steady-state mRNA specific for iNOS (via Northern blot analysis) and increased immunofluorescence for iNOS after cell permeabilization, incubation with anti-iNOS antibody, and treatment with Cy3.18-conjugated rabbit-specific antibody. The extent of inflammatory mediator-induced .NO production by alveolar epithelium, which exceeds that of other lung cell types, reveals new insight into mechanisms of pulmonary host defense and pathways of free radical-mediated lung injury.

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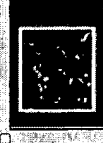
Am. J. Physiol: Lung Cellular and Molecular Physiology

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S. Matalon, K. M. Hardiman, L. Jain, D. C. Eaton, M. Kotlikoff, J. P. Eu, J. Sun, G. Meissner, and J. S. Stamler  
**Regulation of ion channel structure and function by reactive oxygen-nitrogen species**

Am J Physiol Lung Cell Mol Physiol, December 1, 2003; 285(6): L1184 - 1189.

[Abstract] [Full Text] [PDF]



Am. J. Physiol: Lung Cellular and Molecular Physiology

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P. O. Scumpia, P. J. Sarcia, V. G. DeMarco, B. R. Stevens, and J. W. Skimming

**Hypothermia attenuates iNOS, CAT-1, CAT-2, and nitric oxide expression in lungs of endotoxemic rats**

Am J Physiol Lung Cell Mol Physiol, December 1, 2002; 283(6): L1231 - 1238.

[Abstract] [Full Text] [PDF]



The Journal of Immunology

HOME

F. Rose, B. Guthmann, T. Tenenbaum, L. Fink, A. Ghofrani, N. Weissmann, P. Konig, L. Ermert, G. Dahlem, J. Haenze, W. Kummer, W. Seeger, and F. Grimminger

**Apical, But Not Basolateral, Endotoxin Preincubation Protects Alveolar Epithelial Cells Against Hydrogen Peroxide-Induced Loss of Barrier Function: The Role of Nitric Oxide Synthesis**

J. Immunol., August 1, 2002; 169(3): 1474 - 1481.

[Abstract] [Full Text] [PDF]



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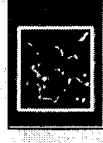
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P. Juvin, T. Fournier, M. Grandsaigne, J.-M. Desmonts, and M. Aubier

**Diesel particles increase phosphatidylcholine release through a NO pathway in alveolar type II cells**

Am J Physiol Lung Cell Mol Physiol, May 1, 2002; 282(5): L1075 - 1081.

[Abstract] [Full Text] [PDF]



Am. J. Physiol: Lung Cellular and Molecular Physiology

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D. V. Pechkovsky, G. Zissel, T. Goldmann, M. Einhaus, C. Taube, H. Magnussen, M. Schlaak, and J. Muller-Quernheim

**Pattern of NOS2 and NOS3 mRNA expression in human A549 cells and primary cultured AEC II**

Am J Physiol Lung Cell Mol Physiol, April 1, 2002; 282(4): L684 - 692.

[Abstract] [Full Text] [PDF]



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F. Rose, G. Dahlem, B. Guthmann, F. Grimminger, U. Maus, J. Hanzé, N. Duemmer, U. Grandel, W. Seeger, and H. A. Ghofrani

**Mediator generation and signaling events in alveolar epithelial cells attacked by *S. aureus* alpha-toxin**

Am J Physiol Lung Cell Mol Physiol, February 1, 2002; 282(2): L207 - 214.

[Abstract] [Full Text] [PDF]



Infection and Immunity

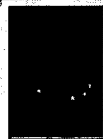
HOME

K. J. Goodrum and J. Poulson-Dunlap

**Cytokine Responses to Group B Streptococci Induce Nitric Oxide Production in Respiratory Epithelial Cells**

Infect. Immun., January 1, 2002; 70(1): 49 - 54.

[Abstract] [Full Text] [PDF]



AMERICAN JOURNAL OF Respiratory Cell and Molecular Biology

HOME

Y. K. Zhu, X. Liu, R. F. Ertl, T. Kohyama, F. Q. Wen, H. Wang, J. R. Spurzem, D. J. Romberger, and S. I. Rennard  
**Retinoic Acid Attenuates Cytokine-Driven Fibroblast Degradation of Extracellular Matrix in Three-Dimensional Culture**

Am. J. Respir. Cell Mol. Biol., November 1, 2001; 25(5): 620 - 627.

[Abstract] [Full Text] [PDF]



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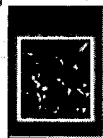
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**Coexpressed Nitric Oxide Synthase and Apical {beta}1 Integrins Influence Tubule Cell Adhesion after Cytokine-Induced Injury**

J. Am. Soc. Nephrol., November 1, 2001; 12(11): 2370 - 2383.

[Abstract] [Full Text] [PDF]



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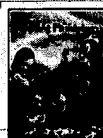
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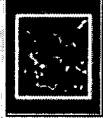
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0193-1857/03 \$5.00

Vol. 284, Issue 3, G373-G384, March 2003

## Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes

Marion Scharte<sup>1,2</sup>, Xiaonan Han<sup>2</sup>, Daniel J. Bertges<sup>3</sup>, Mitchell P. Fink<sup>2,3</sup>, and Russell L. Delude<sup>2,4</sup>

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Cellular adaptation to hypoxia depends, in part, on the transcription factor hypoxia-inducible factor-1 (HIF-1). Normoxic cells exposed to an inflammatory milieu often manifest phenotypic changes, such as increased glycolysis, that are reminiscent of those observed in hypoxic cells. Accordingly, we investigated the effects of cytomix, a mixture containing IFN- $\gamma$ , TNF, and IL-1 $\beta$  on the expression of HIF-1-dependent proteins under normoxic and hypoxic conditions. Incubation of intestine-derived epithelial cells (IEC-6) under 1% O<sub>2</sub> increased HIF-1 DNA binding and expression of aldolase A, enolase-1, and VEGF mRNA.

Incubation of normoxic cells with cytomix for 48 h also markedly increased HIF-1 DNA binding and expression of mRNAs for these proteins. Incubation of hypoxic cells with cytomix did not inhibit HIF-1 DNA binding or upregulation of HIF-1-dependent genes in response to hypoxia. Neither cytomix nor hypoxia increased steady-state levels of HIF-1 $\alpha$  mRNA. Incubation of IEC-6 cells with cytomix induced nitric oxide (NO $\cdot$ ) biosynthesis, which was blocked if the cultures contained L-N<sup>G</sup>-(1-iminoethyl)lysine hydrochloride (L-NIL). Treatment with L-NIL, however, failed to significantly alter aldolase A, enolase-1, and VEGF mRNA levels in normoxic cytomix-treated cells. Proinflammatory cytokines activate the HIF-1 pathway and increase expression of glycolytic genes in nontransformed rat intestinal epithelial cells, largely through an NO $\cdot$ -independent mechanism.

nitric oxide; glycolysis; epithelium; intestinal; aldolase A; enolase-1; vascular endothelial growth factor; hypoxia-inducible factor-1; deoxyribonucleic acid

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## Proinflammatory cytokines increase the rate of glycolysis and adenosine-5'-triphosphate turnover in cultured rat enterocytes.

Berg S, Sappington PL, Guzik LJ, Delude RL, Fink MP.

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**OBJECTIVE:** Measurements of steady-state adenosine-5'-triphosphate (ATP) levels in tissue samples from patients or experimental animals with sepsis or endotoxemia provide little information about the rate of ATP production and consumption in these conditions. Accordingly, we sought to use an in vitro "reductionist" model of sepsis to test the hypothesis that proinflammatory cytokines modulate ATP turnover rate. **DESIGN:** In vitro "reductionist" model of sepsis. **SETTING:** University laboratory. **SUBJECTS:** Cultured rat enterocyte-like cells. **INTERVENTIONS:** IEC-6 nontransformed rat enterocytes were studied under control conditions or following incubation for 24 or 48 hrs with cytomix, a mixture of tumor necrosis factor-alpha (10 ng/mL), interleukin-1beta (1 ng/mL), and interferon-gamma (1000 units/mL). To measure ATP turnover rate, ATP synthesis was acutely blocked by adding to the cells a mixture of 2-deoxyglucose (10 mM), potassium cyanide (8 mM), and antimycin A (1 microM). ATP content was measured at baseline (before metabolic inhibition) and 0.5, 1, 2, 5, and 10 mins later. Log-linear ATP decay curves were generated and the kinetics of ATP utilization thereby calculated. **MEASUREMENTS AND MAIN RESULTS:** ATP consumption rate was higher in cytomix-stimulated compared with control cells (3.11 +/- 1.39 vs. 1.25 +/- 0.66 nmol/min, respectively;  $p < .01$ ). Similarly, the half-time for ATP disappearance was shorter in cytomix-stimulated compared with control cells (2.63 +/- 1.00 vs. 6.21 +/- 3.49;  $p < .05$ ). In contrast to these findings, the rate of ATP disappearance was similar in cytokine-naïve and immunostimulated IEC-6 cells when protein and nucleic acid synthesis were inhibited by adding 50 microg/mL cycloheximide and 5 microg/mL actinomycin D to cultures for 4 hrs. The rates of glucose consumption and lactate production were significantly greater in cytomix-stimulated compared with controls cells. **CONCLUSIONS:** Incubation of IEC-6 cells with cytomix significantly increased ATP turnover. Increased ATP turnover rate was supported by increases in the rate of anaerobic glycolysis. These findings support the view that proinflammatory mediators impose a metabolic demand on visceral cells. In sepsis, cells may be more susceptible to dysfunction on the basis of diminished oxygen delivery and/or mitochondrial dysfunction.

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## Cytokine Responses to Group B Streptococci Induce Nitric Oxide Production in Respiratory Epithelial Cells

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### ▶ ABSTRACT

*Streptococcus agalactiae* (group B streptococcus [GBS]) is a leading cause of neonatal pneumonia, sepsis, and meningitis. Early-onset GBS pneumonia is characterized by marked pulmonary epithelial and endothelial cell injury. Innate proinflammatory responses to GBS infection that may contribute to the respiratory pathology include the synthesis and release of cytokines, prostaglandins, and nitric oxide (NO). The hypothesis that NO is directly induced in lung epithelial cells by invading GBS or indirectly induced by cytokines released by GBS-infected mononuclear cells was tested. A549 transformed human respiratory epithelial cells were directly cultured with GBS, cocultured with GBS-infected human mononuclear cells or purified macrophages, or exposed to conditioned culture medium from human mononuclear cells infected by GBS. The culture medium of A549 cultures was assayed for NO secretion, and the cell lysates were tested for presence of inducible nitric oxide synthase (iNOS) mRNA by reverse transcriptase PCR (RT-PCR). GBS-treated A549 cells neither secreted detectable NO nor expressed iNOS mRNA. GBS interaction with human mononuclear cells, however, stimulated release of soluble factors that readily induced iNOS mRNA expression and NO secretion by A549 cells. Inflammatory mediator-induced nitric oxide (NO) production by alveolar epithelium may exceed that of other lung cell types such as macrophages, and induction during GBS infection may play a significant role in pulmonary defense or free-radical-mediated lung injury.

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### ▶ INTRODUCTION

*Streptococcus agalactiae* (group B streptococcus [GBS]) is a leading cause of neonatal pneumonia, sepsis, and meningitis (26). GBS is also an important pathogen in maternal infections and in infections of nonpregnant adults with risk factors such as older age and underlying disorders (25). Early-onset GBS pneumonia is characterized by presence of numerous bacteria, an inflammatory exudate, and marked pulmonary epithelial and endothelial cell injury (1, 10). Innate proinflammatory responses to GBS infection which may contribute to the respiratory pathology include the synthesis and release of cytokines (12, 22), prostaglandins (16), and nitric oxide (NO) (6, 15).

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In the lung, alveolar macrophages, airway epithelial cells, endothelial cells, and inflammatory cells may be sources for NO (24). Though GBS, with or without cytokines, has been shown to induce NO production in cultured murine macrophages (3, 6, 20), human macrophages produce far less NO (5- to 100-fold less) in response to cytokine or microbial stimuli than do murine macrophages (31). An alternate source of alveolar NO are human lung epithelial cells, which possess a nitric oxide synthase (iNOS, NOS2) that is inducible by a mixture of cytokines (21) that should, based on in vitro data, be released locally by GBS-infected alveolar macrophages and mononuclear cells (12). Alternatively, direct GBS invasion of respiratory epithelial cells (23) might also induce NO production.

In this report, human respiratory epithelial cells were directly cultured with GBS, cocultured with GBS-infected mononuclear cells, or exposed to conditioned culture medium from human mononuclear cells infected by GBS. The culture medium of epithelial cell cultures was assayed for nitrite, and the cell lysates were tested for iNOS mRNA by reverse transcription-PCR (RT-PCR). Whereas direct GBS infection of epithelial cells failed to induce NO, conditioned medium from GBS-treated mononuclear cells stimulated both NO secretion and iNOS expression by respiratory epithelial cells.

## ► MATERIALS AND METHODS

**Bacteria.** The highly encapsulated serotype III GBS strain COH1 and the isogenic mutant of COH1, COH1-13 (lacking type III polysaccharide), were provided by Craig E. Rubens (University of Washington School of Medicine, Seattle). GBS were harvested by centrifugation from log-phase cultures in Todd-Hewitt broth and washed in phosphate-buffered saline (PBS). GBS were enumerated by viable colony counts on blood agar, and equivalent doses of GBS strains were adjusted by optical density comparisons of bacterial suspensions.

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**Reagents.** Polymyxin B, gentamicin, and penicillin G were obtained from Sigma Chemical Co. (St. Louis, Mo.). Recombinant human interleukin-1- $\beta$  (IL-1), tumor necrosis factor alpha (TNF- $\alpha$ ), and gamma interferon (IFN- $\gamma$ ) were purchased from Biosource International (Camarillo, Calif.).

**Respiratory epithelial cells.** Primary cultures of normal human bronchial epithelial (NHBE) cells were obtained from Clonetics Cell Systems (BioWhittaker, Walkersville, Md.) and cultured in serum-free medium provided by Clonetics. The A549 human lung carcinoma cell line (ATCC, CCL185) was routinely

cultured in Dulbecco modified Eagle medium (DMEM) with 4,500 mg of glucose/liter (HyClone, Logan, Utah) with or without 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), plus 100 U of penicillin G and 100 µg of streptomycin/ml.

**GBS infection of epithelial cells.** Epithelial cells were cultured in antibiotic-free medium 24 h prior to infection. Bacterial invasion of epithelial cell cultures was initiated as described by Rubens et al. (23) by centrifugation (800 x g, 10 min) of viable washed log-phase GBS (doses from 1 to 1,000 CFU of GBS/epithelial cell) directly onto adherent epithelial cells in multiwell culture plates. After a 2-h invasion period at 37°C, culture wells were washed four times with PBS to remove excess extracellular bacteria, and medium with antibiotics (100 µg of gentamicin and 5 µg of penicillin G/ml) was added to kill the remaining extracellular bacteria. Invasion periods of up to 4 h were tested but resulted in a loss of epithelial cell viability. Culture medium and/or cell lysates were collected at timed intervals from replicate culture wells for NO assay or RNA extraction.

**Nitrite assay.** NO synthesis was determined colorimetrically as the accumulation of nitrite ( $\text{NO}_2^-$ ) in culture medium. Briefly, 100-µl aliquots of conditioned medium were mixed with 100 µl of Greiss reagent (1:1 [vol/vol] 0.02% *N*-[1-naphthyl]ethylenediamine dihydrochloride [Sigma, St. Louis, Mo.] in  $\text{H}_2\text{O}$ -1% sulfanilamide in 3 N HCl) in flat-bottom, 96-well immunoassay plates (Falcon; Becton-Dickinson, Oxnard, Calif.). After a 20-min incubation at room temperature, the  $A_{540}$  was measured on a microplate reader (Bio-Tek Instruments, Winooski, Vt.). The nitrite concentration was determined from a standard curve generated with sodium nitrite ( $\text{NaNO}_2$ ). For samples with marginally detectable or undetectable nitrite levels, samples were incubated with nitrate reductase (from corn seedling [Sigma], 0.01 U/sample) plus NADH (0.2 mM final concentration) to convert nitrate by-products to nitrite prior to the Greiss reaction.

**iNOS mRNA expression.** For RNA extraction, epithelial cells were cultured in six-well cluster plates or T25 culture flasks, and total RNA was extracted by using the RNAgents System (Promega Corp., Madison, Wis.) or RNA Stat-60 reagent (Tel-Test, Inc., Friendswood, Tex.). RNA pellets were suspended in RNase-free water and frozen at -135°C. iNOS mRNA was detected by RT-PCR. A one-tube RT-PCR (Access RT-PCR System; Promega) was used as described by the manufacturer. To eliminate contaminating DNA, extracted RNA was DNase treated (1 U of RQ1 RNase-free DNase per µg of RNA, followed by DNase stop solution, as described by the manufacturer [Promega]). The DNase digestion reaction was followed by RT-PCR. RNA samples (1 µg) were used based on concentrations of extracted RNA estimated by optical density (260 nm/280 nm). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers, IL-8 primers, and human iNOS primers were obtained from Clontech (Palo Alto, Calif.). As an additional control for variations in RNA quantity or quality, an Ambion (Austin, Tex.) human iNOS relative RT-PCR kit was also used. This multiplex PCR uses two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competitors as an invariant endogenous control). Optimal PCR products were obtained with 1.5 mM magnesium. The reaction incubation cycles were as follows: 1 cycle (48°C for 45 min), 1 cycle (94°C for 2 min), 40 cycles (94°C for 30 s, 60°C for 1 min, and 68°C for 2 min), and 1 cycle (68°C for 7 min). Reaction cycles were controlled by a thermal cycler (Easy Cycler; Ericomp, San Diego, Calif.). PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel.



**PBMC.** Human peripheral blood mononuclear cells (PBMC) were purified from venous blood from healthy adult volunteers. The use of human subjects was reviewed by the Ohio University Institutional Review Board and complied with all relevant federal guidelines and institutional policies. Blood samples (50 ml in EDTA-containing blood collection tubes) were layered over equal volumes of Histopaque 1077 (Sigma) at room temperature, and the preparations were centrifuged (400 x g, room temperature) for 30 min. The mononuclear cell fraction was collected and washed three times (250 x g, 10 min, room temperature) in PBS plus 2% glucose to remove most platelets and suspended in 5 ml of supplemented RPMI medium (50:50 RPMI 1640-DMEM), plus 10 mM HEPES, penicillin G (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 0.1% glucose, 0.006% L-arginine, and 5% heat-inactivated human type AB serum (Irvine Scientific, Irvine, Calif.). Viable total mononuclear cell counts were performed by using trypan blue. Differential cell counts were performed by using Hema 3 Wright stain (Fisher Scientific, Pittsburgh, Pa.).

**Cocultures.** Mononuclear cell suspensions containing  $9 \times 10^5$  monocytes in 1.5 ml of supplemented RPMI were added for 1 h in 24-mm Transwell-Clear polyester membrane inserts (0.4-µm pore size; Corning Costar, Cambridge, Mass.). The lower six-well culture chambers contained 2.6 ml of supplemented medium. Culture inserts with PBMC or with adherent culture-matured macrophages (see below) were placed in wells of confluent A549 cells. A549 cells were grown in DMEM and then switched to supplemented medium when inserts were added. Washed GBS at a ratio of 1:1 or 10:1 to the monocyte/macrophage count were added to the inserts. Cocultures were incubated for 24 h. A549 cell chamber medium and macrophage insert medium were collected and frozen at -20°C for nitrite assays. Total RNA was extracted from A549 cells and stored at -135°C.

To prepare inserts enriched for macrophages, nonadherent mononuclear cells were aspirated from culture inserts 1 h after addition of the washed mononuclear cell fraction, followed by two washes with PBS plus 2% glucose and the addition of fresh medium. Adherent monocytes were washed again after 24 h and cultured for 6 to 7 days at 37°C in 5% CO<sub>2</sub> for maturation into macrophages. The medium was changed every 72 h.

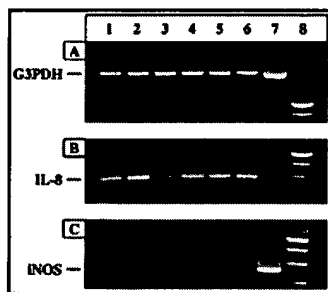
**Collection of conditioned medium from mononuclear cells for treatment of A549 cells.** A total of  $5 \times 10^6$  mononuclear cells/well in supplemented medium (2 ml/well) was added to six-well multiwell tissue culture plates. Washed GBS were added at a ratio of 1:1 or 10:1 as described above, and cultures were incubated at 37°C. Conditioned medium was collected at 24 h and centrifuged to remove cells and bacteria. A small sample of the conditioned medium was frozen for future assay of nitrite levels. The remainder of the medium was transferred to confluent A549 cell monolayers, which had been grown in supplemented medium in other six-well plates. After an additional 24 h of incubation at 37°C, the culture medium was collected for nitrite assays, and the total RNA was extracted from the A549 cell monolayer. To generate larger samples of A549 RNA, for some experiments 5 ml of conditioned medium (from  $1.4 \times 10^7$  mononuclear cells in a T25 tissue culture flask) was collected and added to confluent A549 cells in a T25 flask. Background nitrate and/or nitrite levels in culture medium were accounted for by diluting nitrite standards for the Greiss assay in the stock culture medium and by using stock culture medium as a background control.

**Statistics.** Means of experimental groups were considered significantly different if the *P* value was  $\leq 0.05$ . Statistical significance was analyzed by comparison of mean values by using the Student's *t* test (unpaired, two tailed).

## RESULTS

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**NO production by direct GBS infection of A549 cells or NHBE cells.** Direct treatment of A549 cells or NHBE cells with living GBS (a 2-h infection pulse with doses of from 1 to 1,000 CFU of COH1 or COH1-13 per epithelial cell) did not induce detectable nitrite accumulation ( $<0.6 \mu\text{M}$  at 24 h in at least five experiments with each cell type) in the culture medium, nor did GBS infection induce iNOS mRNA (at 2 or 24 h) in A549 cells as detected by RT-PCR. A barely visible iNOS PCR product was seen in both untreated and GBS-treated (2 h) NHBE cells when NHBE cells (two lots) were tested in their first culture passage from receipt as frozen cells. NHBE cells may perhaps become transiently activated when collected from donors. In subsequent passages, GBS-treated NHBE cells did not exhibit iNOS expression (Fig. 1). Both NHBE and A549 cells were viable after GBS treatment and responsive to GBS, as shown by secretion and accumulation for 24 h in culture medium of immunoreactive IL-8 (data not shown; K. J. Goodrum, unpublished data) and induction of IL-8 mRNA (2 h, Fig. 1). As a control that epithelial cells could be induced to express iNOS under the conditions and timing of the cultures, A549 cells were treated for 24 h with a mixture (CytoMix) of IL-1, TNF- $\alpha$ , and IFN- $\gamma$  (10 ng/ml each). CytoMix-treated A549 cells secreted NO (a mean nitrite level in 24-h culture medium of  $6.4 \pm 4.3 \mu\text{M}$  in four experiments) and expressed iNOS mRNA detectable by RT-PCR at 2 h posttreatment, with higher expression detectable at 24 h (Fig. 2).



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**FIG. 1.** Direct GBS infection of NHBE cells induces IL-8 but not iNOS mRNA. Total RNA was purified from cell lysates collected at 2, 8, and 24 h after initiation of a 2-h infection pulse with COH1-13 (100:1). RT-PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel. (Gel A) G3PDH RT-PCR products (G3PDH PCR product = 983 bp). (Gel B) IL-8 RT-PCR products (IL-8 PCR product = 289 bp). (Gel C) iNOS RT-PCR products (iNOS PCR product = 259 bp). Lanes 1, 3, and 5, uninfected cells at 2, 8, and 24 h, respectively; lanes 2, 4, and 6, GBS-infected cells at 2, 8, and 24 h, respectively; lane 7, control RNA for respective genes; lane 8, DNA molecular weight markers.



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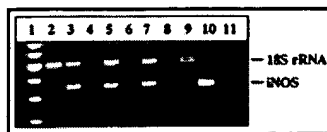
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**FIG. 2. Cytomix induces iNOS mRNA in A549 cells.** A549 cells were treated for 2 or 24 h with 10 ng each of IL-1, TNF- $\alpha$ , and IFN- $\gamma$ /ml. Total RNA was extracted from cell lysates, and multiplex RT-PCR was conducted with two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competitors as an invariant endogenous control). PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel. Lane 1, DNA markers; lane 2, untreated (2 h) A549; lane 3 and 5, Cytomix-treated (2 h) A549; lane 4, same as lane 3 but without reverse transcriptase; lane 6, untreated (24 h) A549; lanes 7 and 9, Cytomix-treated (24 h) A549; lane 8, same as lane 7 but without reverse transcriptase; lane 10, 18S rRNA control (495 bp); lane 11, iNOS RNA control (349 bp).

**iNOS gene expression in A549 cells cocultured with GBS-treated PBMC.** A549 cells are known to express iNOS when treated with a mixture of IL-1, TNF- $\alpha$ , and IFN- $\gamma$  (Cytomix) but poorly (14) or not at all (21) in response to the doses of any single cytokine in this mixture. Since GBS-treated PBMC are known to release all of these cytokines (12), indirect induction of iNOS was examined in A549 cells cocultured (using culture inserts) with human PBMC or culture-derived human macrophages. In addition to testing cocultures of A549 and PBMC, separate experiments tested the iNOS response of A549 cells when cultured in the presence of conditioned medium from separately cultured PBMC. Based on the timing of detectable induced NO levels and iNOS mRNA in Cytomix-treated A549 cells, samples from coculture experiments were collected at 24 h.

Secreted NO in the culture medium and iNOS gene expression were undetectable in untreated A549 cultures, A549 cultures treated directly with GBS, A549 cocultures with uninfected macrophage or PBMC, and A549 cocultures with only GBS-inoculated culture medium in the culture insert. Nitrite was not detectable in conditioned medium from GBS-treated PBMC or GBS-treated macrophages. Only when A549 cells were cocultured with GBS-treated PBMC or were treated with supernatant fluid collected from separately cultured GBS-treated PBMC was nitrite accumulation in the medium (mean  $\pm$  the standard deviation of five experiments =  $2.8 \pm 1.9$   $\mu$ M at a GBS [strain COH1]/PBMC treatment ratio of 1:1) and iNOS gene expression readily detectable (Fig. 3 and 4). Culture medium supernates from PBMC treated at a GBS/mononuclear cell treatment ratio of 1:1 reproducibly induced NO and iNOS in A549 cells in five experiments with mononuclear cells from five different donors. Induction of iNOS mRNA with a GBS (strain COH1)/mononuclear cell treatment ratio of 10:1 was found in four of four experiments; however, NO production (nitrite accumulation in the medium) was detected in only two of four experiments at this dose. Conditioned medium generated with either COH1 or with the isogenic nonencapsulated mutant strain COH1-13 (1:1 treatment ratio of GBS/mononuclear cell) was effective at inducing NO production in A549 cells (mean value from combined experiments with two donors [duplicate cultures of each] of  $4.4 \pm 1.7$  and  $3.7 \pm 1.2$   $\mu$ M nitrite with COH1 versus COH1-13, respectively).

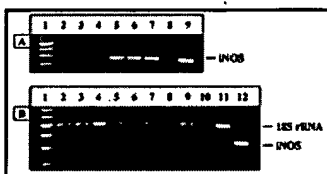


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**FIG. 3.** iNOS mRNA induction in A549 cells treated with conditioned medium from GBS-treated human mononuclear cells. We centrifuged 24-h-conditioned medium from GBS-treated (strain COH1, 1:1 or 10:1 ratio) human mononuclear cells to remove GBS and added it to monolayers of A549 cells for 24 h. Total RNA was extracted from cell lysates, and multiplex RT-PCR was conducted with two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competitors as an invariant endogenous control). PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel. Lane 1, DNA markers; lane 2, A549 cells treated with conditioned medium from control mononuclear cells (uninfected); lanes 3 and 5, A549 cells treated with conditioned medium from GBS (1:1)-infected mononuclear cells; lanes 4 and 6, same as lanes 3 and 5 but without reverse transcriptase; lane 7, A549 cells treated with conditioned medium from GBS (10:1)-infected mononuclear cells; lane 8, same as lane 7 but without reverse transcriptase; lane 9, 18S rRNA control (495 bp); lane 10, iNOS RNA control (349 bp).



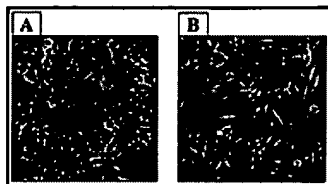
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**FIG. 4.** iNOS mRNA induction in A549 cells cocultured with GBS-treated human mononuclear cells. A total of  $5 \times 10^6$  mononuclear cells treated with GBS strain COH1 (1:1 ratio) were placed in 24-mm Costar Transwell culture inserts. Inserts were cocultured with monolayers of A549 cells on Transwell multiwell plates for 24 h. Total RNA was extracted from A549 cell lysates, and RT-PCR was conducted. PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel. (Gel A) RT-PCR products with Clontech iNOS primers. Lane 1, DNA markers; lane 2, A549 cocultured with uninfected mononuclear cells; lane 3, A549 cocultured with GBS in insert (without mononuclear cells); lane 4, A549 only (no coculture); lanes 5 and 6, A549 cocultured with GBS treated (1:1) mononuclear cells; lane 7, A549 cocultured with GBS-treated (10:1) mononuclear cells; lane 8, no RNA added; lane 9, iNOS RNA control (259 bp). (Gel B) The same samples as in gel A were reanalyzed with multiplex RT-PCR. Lanes 1 to 5 were as described above. Lane 6, same as lane 5 but without reverse transcriptase; lanes 7 and 8, duplicates of lanes 5 and 6; lane 9, A549 cocultured with GBS-treated (10:1) mononuclear cells; lane 10, same as lane 9 but without reverse transcriptase; lane 11, 18S rRNA control (495 bp); lane 12, iNOS RNA control (349 bp).

**Changes in epithelial cell morphology.** Whereas the relationship to iNOS induction is unknown, A549 cells cocultured with GBS-treated PBMC or treated with supernatant fluid collected from GBS-treated human PBMC exhibited a marked morphologic change. A549 monolayers thus treated lost their cuboidal arrangement and became very elongated with dendritic processes (Fig. 5).



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**FIG. 5.** Morphological change in A549 cells treated with conditioned medium from GBS-treated human mononuclear cells. We centrifuged 24-h-conditioned medium from GBS-treated (strain COH1, 1:1) human mononuclear cells to remove GBS and then added it to monolayers of A549 cells for 24 h. (A) A549 cells treated with conditioned medium from control untreated mononuclear cells. (B) A549 cells treated with conditioned medium from GBS-treated mononuclear cells. Both panels are at the same magnification.

**Role of macrophages in cocultures.** In an attempt to determine whether macrophages were the source of the soluble factors from PBMC, coculture experiments were conducted with adherent culture-derived macrophages. Neither NO secretion nor iNOS gene expression was found in A549 cocultured with GBS-treated adherent culture-derived human macrophages, indicating that macrophages alone do not produce the factor or combination of factors responsible. Since GBS-stimulated human monocytes/macrophages are known to produce two (IL-1 and TNF- $\alpha$ ) of the three components in Cytomix, the A549-macrophage coculture experiment was repeated with the addition of the third Cytomix component (10 ng of human IFN- $\gamma$ /ml). IFN-treated A549-macrophage cocultures also failed to release NO or express iNOS in response to GBS treatment (three experiments). This indicates that the factor(s) released in PBMC cocultures differs quantitatively or qualitatively from the Cytomix signal.

## DISCUSSION

Innate immune responses to GBS within the respiratory tract may be initiated directly via invasion of respiratory epithelial cells and via phagocytosis by alveolar macrophages. In addition, the numerous proinflammatory factors released by infected epithelial and phagocytic cells may reciprocally modulate each other's functions as well as recruit inflammatory cells and modulate the functions of other resident respiratory cells. GBS, a respiratory pathogen in neonates, is shown here to have no direct effect on iNOS expression when infecting human respiratory epithelial cells. GBS interaction with human mononuclear cells, however, stimulates the release of soluble factors that readily induce iNOS expression in human respiratory epithelial cells.

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NO plays important roles in both physiological and pathological responses to infection (32). NO mediates proinflammatory actions by increasing blood flow and vascular permeability as well as mediating cell and tissue injury and potent antimicrobial activity (32). Coincident production of NO and superoxide ( $O_2^-$ ) promotes formation of peroxynitrite ( $ONOO^-$ ), an important factor in tissue injury at sites of inflammation (24). NO production in animal models of GBS infection has been shown to enhance meningeal inflammation (8), to mediate neuronal injury in brain cells (11), and to destroy lung surfactant (3). On the other hand, enhanced NO levels mediate beneficial hemodynamic effects in GBS infection by reducing pulmonary hypertension (2) and cerebral ischemia (15). The role of NO in septic shock and pulmonary hypertension in GBS infections of humans is controversial (5, 27).

Inducible NOS is present in a variety of cell types, including macrophages and respiratory epithelial cells (19), and transcription is the major step in regulation of iNOS mRNA (19, 32). Optimal expression of iNOS in human or rat respiratory epithelial cells requires synergistic signals from combinations of cytokines (14, 21) or lipopolysaccharide (LPS) plus cytokines (7). Whereas treatment of A549 cells with GBS alone or individual cytokines alone did not induce iNOS, GBS-induced soluble factors, presumably cytokines, from mononuclear cells did induce both iNOS and secreted NO in A549 cells. Since GBS alone does not induce iNOS mRNA in A549 cells, GBS passage between coculture chambers as reported in studies of GBS transcytosis of endothelial cell monolayers (18) would not explain the induction of iNOS mRNA. Possible synergy between GBS and individual cytokines or combinations of cytokines was not tested. Though bacterium-free culture supernatants from mononuclear cells were used to stimulate the A549 cells, it is possible that soluble or released bacterial factors acted in synergy with cytokines to induce iNOS. The GBS beta-hemolysin has been reported to induce iNOS in the RAW264.7 mouse macrophage cell line (20), as well as exhibit toxicity toward the A549 cell line (17). An investigation of epithelial responses to additional GBS serotypes and strains is necessary to fully resolve the direct versus indirect effects of GBS. Results similar to those reported here for A549 responses to GBS have been reported with other bacterial stimuli. Conditioned medium from tubercle bacillus-treated human mononuclear cells, more than conditioned medium from LPS-treated mononuclear cells, induced iNOS mRNA and NO production in A549 cells (13).

The GBS-induced soluble factor(s) involved in iNOS induction were not identified, but the known responsiveness of A549 cells to a mixture of IL-1, TNF- $\alpha$ , and IFN- $\gamma$  would support a hypothesis for GBS induction of these cytokines. GBS-treated human monocytes can produce IL-1, IL-6, and TNF- $\alpha$  (30), whereas GBS-induced IL-12 from macrophages can innately induce IFN- $\gamma$  from NK cells (4). The finding that GBS-treated macrophages failed to signal iNOS induction in A549 cocultures even when supplemented with IFN- $\gamma$  argues against this hypothesis, but the relative concentrations of macrophage cytokines in these cocultures may be critical and were not specifically measured. Conditioned medium from GBS-treated human PBMC, which have been shown to produce IL-1, TNF- $\alpha$ , IFN- $\gamma$ , and IL-12 (12), did induce iNOS in A549 cocultures. One or more of these or other unexamined factors secreted by GBS-treated PBMC must be modulating iNOS expression and/or cellular morphology in the A549 cells. Although many investigators have confirmed that NHBE cells and A549 cells (7, 14, 21) have parallel iNOS responses to Cytomix, the stimulatory effect of GBS-treated PBMC on iNOS induction in A549 cells was not examined in NHBE cells.

A549 cells cultured with conditioned medium from PBMC treated with the higher dose of GBS (10:1) consistently expressed iNOS mRNA but did not consistently show elevated nitrite in the medium. The higher GBS dose may have been toxic to the mononuclear cells, possibly inducing a set of factors quantitatively or qualitatively different from those induced at the lower dose (1:1) or generating conditioned medium with factors that induced iNOS but inhibited iNOS activity.

The cause of the morphologic changes seen in A549 cells treated with GBS-conditioned medium from PBMC is unknown. Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) has been reported to transform the morphologic phenotype of A549 cells in a fashion similar to that reported here (28). In addition, A549 cells cocultured with macrophages activated by LPS and IFN- $\gamma$  are reported to exhibit a nitric oxide-mediated

increase in synthesis and activation of latent TGF- $\beta$ 1 protein (29).

Experiments to determine specific cytokine levels in the conditioned medium from GBS-treated PBMC and experiments to test the effects of cytokine neutralization on A549 responses will be required to identify the factor or factors involved in the epithelial responses. Considering the importance of GBS infections in neonates and the reports of reduced cytokine responses to GBS in cord blood versus adult mononuclear cells (9), the studies described here should be repeated with human cord blood cells in order to assess the relevance of this pathway for iNOS induction in newborns. Production of NO via iNOS induction either in respiratory epithelial cells or in vascular endothelial cells in GBS infection may restrict intracellular survival and transcytosis of GBS, thus serving as an innate defense against sepsis and meningitis.

Inflammatory mediator-induced NO production by alveolar epithelium exceeds that of other lung cell types, such as macrophages (7), and induction during GBS infection may play a significant role in pulmonary defense or free-radical-mediated lung injury in GBS disease.

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## FOOTNOTES

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